

Biomarker Strategies to Evaluate the Environmental Effects of Chemicals

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Environmental risk assessment of chemicals depends on the production of toxicity data for surrogate species of mammals, birds, and fish and on making comparisons between these and estimated or predicted environmental concentrations of the chemicals. This paper gives an overview of biomarker assays and strategies that might be used as alternatives, that is, to replace, reduce, or refine currently used ecotoxicity tests that cause suffering to vertebrates. In the present context a biomarker is a biologic response to an environmental chemical at the individual level or below which demonstrates a departure from normal status. Of immediate interest and relevance are nondestructive assays that provide a measure of toxic effect in vertebrate species and that can be used in both laboratory and parallel field studies. A major shortcoming of this approach is that such assays are currently only available for a limited number of chemicals, primarily when the mode of action is known. Nondestructive assays can be performed on blood, skin, excreta, and eggs of birds, fish, reptiles, and amphibians. An interesting recent development is the use of vertebrate cell cultures, including transgenic cell lines that have been developed specifically for toxicity testing. The ultimate concern in ecotoxicology is the effects of chemicals at the level of populations and above. Current risk assessment practices do not address this problem. The development of biomarker strategies could be part of a movement toward more ecologic end points in the safety evaluation of chemicals, which would effect a reduction in animal tests that cause suffering. — *Environ Health Perspect* 106(Suppl 2):613–620 (1998). <http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-2/613-620walker/abstract.html>

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Introduction

During the last 50 years great advances have been made in the field of analytical chemistry. The development and refinement of techniques such as gas chromatography, mass spectroscopy, and atomic absorption has facilitated the detection and determination of ever smaller residues of organic and inorganic pollutants in air, water, soil, sediments, and biota. Thus there has been a growing awareness of the widespread contamination of the environment by a wide variety of chemicals because of the activities of humans. Although the levels of chemicals have usually been very low, reflecting the increasing sensitivity and sophistication of analytical instruments, the complexity of pollution patterns has also

been evident. In response to political pressures arising from mounting public concern, a great deal of analytical work has been done on environmental samples, and there now exists a huge amount of data on environmental levels of organic and inorganic chemicals. There is, however, one fundamental problem: very little of this data can be interpreted in biologic terms. The harmful effects of these chemicals on living organisms remain largely unknown.

Central to the disciplines of toxicology and ecotoxicology is the concept of dose response. The interpretation of residue data from the field depends on establishing relationships between environmental concentrations and harmful effects on living

organisms, which is the principle on which the biomarker approach is based (1–4). This paper will review the application of biomarker strategies in the field, noting the difficulties in comparison of dose–response data from the laboratory with dose–response relationships measured in the field. Destructive biomarker assays for vertebrates will be mentioned only in passing. Emphasis will be given to nondestructive assays for vertebrates and to assays using invertebrates.

Concepts

The Biomarker Concept

In this paper a biomarker is defined as a biologic response to an environmental chemical at the individual level or below which demonstrates a departure from normal status (5). The response may be at the molecular, cellular, or whole-organism level. Changes at the level of population, community, or ecosystem are not included in this definition, although they are the ultimate concern of ecologists when applying the biomarker concept. The relationship between biomarker responses of individual organisms and consequent effects at the levels of populations and above will be discussed later. The biomarker concept is illustrated in Figure 1 (3). In Figure 1A, a relationship is shown between increasing exposure to a chemical and the consequent effects. Exposure in this case refers to the internal concentration of a chemical. It may represent, for example, the increasing tissue concentration with time when there is continuous exposure to a constant concentration of a chemical in food, ambient water, or air. The horizontal axis measures physiologic state and the vertical axis measures health status. Where levels of chemical are sufficiently low, there is no disturbance of homeostasis and the organism remains healthy. As the concentration rises, however, the organisms become stressed and must expend energy in defense mechanisms (e.g., induction of enzymes or metallothioneins) in an attempt to reduce the cellular level of chemical. If the level of chemical rises further, toxic damage occurs and the organism will enter into a reversible diseased state. At this stage stress proteins may be released to repair cellular damage. Finally, a further increase in concentration will lead to an irreversible disease state and the organism will die shortly thereafter.

In Figure 1B, a number of biomarker responses are shown that measure different

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Abbreviations used: Ah receptor, aryl hydrocarbon receptor; B[a]P, benzo[a]pyrene; CALUX, chemically activated luciferase gene expression; ELISA, enzyme-linked immunosorbent assay; OP, organophosphorus; PAH, polycyclic aromatic hydrocarbon; PCB, polychlorinated biphenyl; *p,p'*-DDE, *p,p'*-dichlorophenyl dichloroethylene; T₄, thyroxine; TBT, tributyl tin.

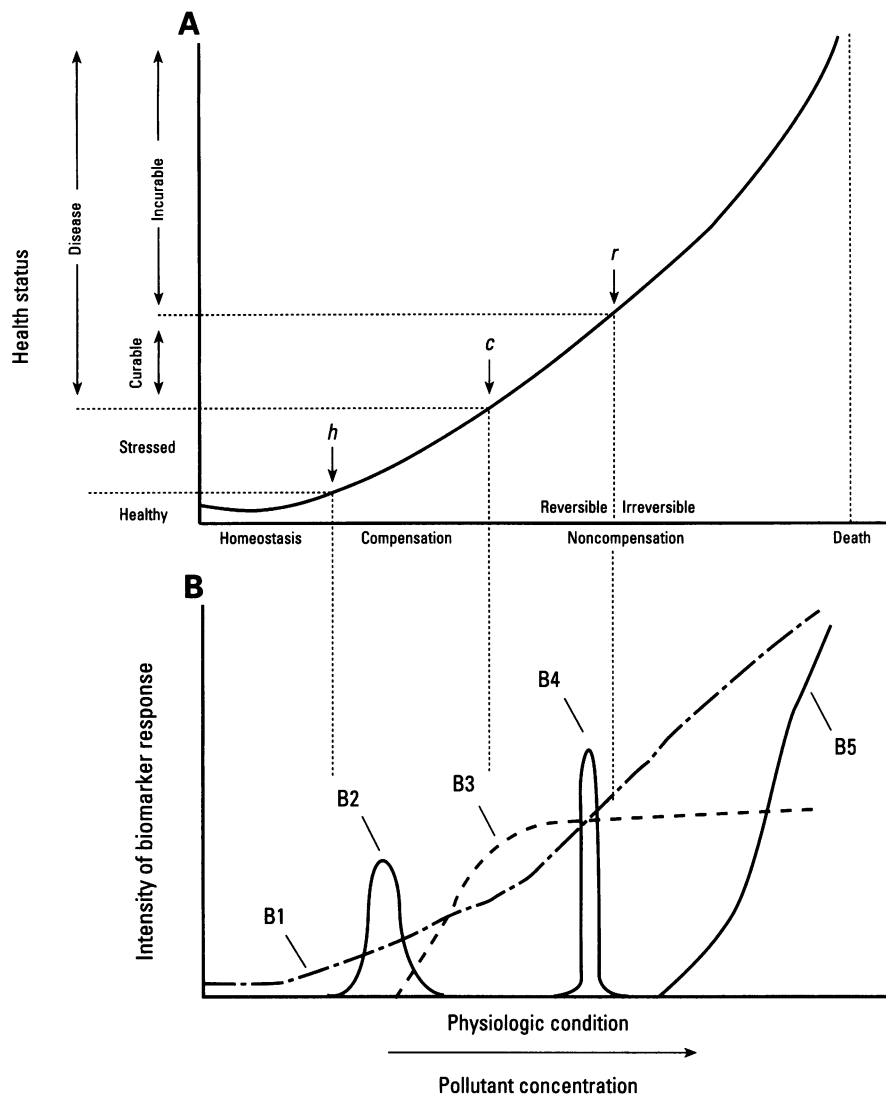


Figure 1. Relationship between exposure to pollutant, health status, and biomarker responses. (A) Progression of the health status of an individual as exposure to pollutant increases: *h*, the point at which departure from the normal homeostatic response range is initiated; *c*, the limit at which compensatory responses can prevent development of overt disease; *r*, the limit beyond which the pathologic damage is irreversible by repair mechanisms. (B) Response of five hypothetical biomarkers used to assess the health of the individual. Reproduced from Walker et al. (7), with permission of Taylor & Francis.

stages in the time-related intoxication process. This is a conceptual diagram and these are hypothetical biomarker responses. The complete diagram does not arise from an integrated experiment performed with a single chemical on a particular organism. However, it does represent the kind of biomarker responses that occur and are therefore not unrealistic. Biomarker A shows a similar relationship to parameters of toxicity that have been well established for the inhibition of the brain acetylcholinesterase of vertebrates by organophosphorus (OP) pesticides (6). With birds, for example, inhibition of up to 40% can be tolerated

without obvious symptoms of toxicity. In the range of approximately 40 to 75%, characteristic physiologic and behavioral effects are seen. Above this range, extreme symptoms of neurotoxicity occur, leading quickly to death. The particular value of this and other biomarker assays based on the mechanism of toxicity will be explained in "Desired Characteristics of Biomarkers." Biomarker B2 could represent the induction of an enzyme with a detoxifying function that is not sustained when the organisms enters the diseased state. Biomarker B3 could represent the release of stress proteins to repair cellular damage

caused by the chemical and biomarker B5 could represent cellular damage (e.g., lysosomal disruption) in the later stages of intoxication. Thus, in principle, an appropriate suite of biomarker assays can monitor the time-related sequence of changes that underlie chemical toxicity, progressing from an initial molecular interaction through cellular disturbances to toxic manifestations at the level of the whole organism. Given the appropriate technology it is possible, in theory, to obtain an integrated and in-depth picture of toxic changes related to dose for organisms living in the natural environment.

Desired Characteristics of Biomarkers

The value of biomarker assays that can monitor the whole sequence of events underlying toxicity has been mentioned. Assays based on molecular mechanisms of toxicity are particularly relevant here because progressive molecular interactions such as cholinesterase inhibitions can cause a sequence of biochemical and physiologic changes leading to toxic symptoms and death. Other examples will be discussed later. Such assays are relatively easy to conceive and even to deliver when the molecular mechanisms of toxicity are known. Unfortunately this knowledge is often lacking. Pesticides represent something of an exception to this rule. Insecticides, for example, frequently have significant vertebrate toxicity and information on mode of action is often required by regulatory authorities in connection with registration. Other biomarker assays that monitor only exposure are less informative when attempting to relate environmental levels of chemicals to toxicity. However, there are ecologic considerations that are frequently overlooked in medical toxicology. The operation of defense mechanisms (e.g., induction of enzymes, Figure 1), although not directly related to toxicity, does involve energy costs. In ecosystems the energy costs borne in this way may have adverse effects on reproduction or growth. Thus there may be harmful effects at the level of population that are a consequence of the operation of defense mechanisms (7). In other words certain biomarkers of exposure for the individual may be biomarkers of harmful effects at the level of population.

Implicit in the present account is the desirability of developing nondestructive biomarkers for vertebrates (8). There are both aesthetic and scientific reasons for this. There is a strong commitment to wildlife

conservation in the Western world, along with growing opposition to the killing of wild vertebrates (9). Also, nondestructive sampling is generally preferable to destructive sampling, as it permits serial sampling of individuals and minimizes the direct influence of the experimenter on populations under investigation in the field.

At a practical and operational level there are four desirable characteristics of the biomarker assay: sensitivity, specificity, simplicity, and stability. An assay should be sensitive enough to detect early stages of the process of toxicity, before the organism reaches the disease state (Figure 1). Such an assay can give early warning of the harmful effects of a pollutant on individuals. Specificity is desirable because it can provide evidence of the harmful effect of a particular type of pollutant and thus evidence of causality if a link is established between the level of a pollutant and adverse effects at the levels of individual and population. Specificity is also limiting, restricting the number of chemicals to which a response can be detected. Simplicity is desirable to make an assay widely available to nonexperts in a cost-effective way. Diagnostic kits such as the enzyme-linked immunosorbent assay (ELISA), which are widely available in medical pathology laboratories, could be developed if there were sufficient demand. Stability is important in the sense that unstable and short-lived responses are difficult to measure and interpret in field studies.

In reality, no biomarker assay exists that has all of these attributes, and it is unlikely that any ever will. On the other hand this limitation can be overcome by using combinations of biomarkers, a strategy that is in any case to be recommended for other reasons (see earlier discussions). Thus, a simple and inexpensive biomarker may be widely used to demonstrate a toxic effect in a population and a small number of individuals from the sampled population may also be assayed by a complex, expensive, specific biomarker to provide evidence of causality (i.e., attribution of effect to a particular pollutant or group of pollutants).

Biomarkers and Interactive Effects

A fundamental problem in the assessment of environmental effects of pollutants is the question of mixtures (7). Not uncommonly, organisms are exposed to complex mixtures of pollutants (e.g., polychlorinated biphenyls [PCBs], polycyclic aromatic hydrocarbons [PAHs], metals), albeit at low concentrations. Toxicity testing is

nearly always restricted to individual compounds; very little work being done on combinations of chemicals. Although seldom used until now, certain biomarkers provide a measure of the harmful effects of mixtures in a nondestructive way (10,11).

In considering individual organisms, the toxicity of a mixture usually approximates to the sum of the toxicities of its individual components. However, the major concern in the present context is potentiation, where toxicity is substantially greater than the sum of individual toxicities. In this case biomarkers that provide a measure of toxicity should establish where there is potentiation (e.g., because of toxicokinetic interactions). Biomarker responses not clearly related to toxicity (e.g., induction of certain detoxification enzymes) do not provide such a measure. The argument is different when considering effects at the level of population and above. Biomarkers of toxic effect as well as biomarkers that relate to energy cost may provide evidence of potentiation for reasons explained in "The Biomarker Concept." However, it has not yet been possible to establish links between such biomarker responses and population effects. In contrast, biomarkers of toxic effect such as eggshell thinning due to *p,p'*-dichloroethylene (*p,p'*-DDE) in birds and imposex due to tributyl tin (TBT) in the dogwhelk (*Nucella lapillus*) have been clearly linked to population declines in field studies (1,7).

Extrapolation from Laboratory to Field

Dose-response relationships between concentrations (doses) of pollutants and biomarker responses can be established under closely controlled conditions in the laboratory. The important question is whether the same or similar responses are observed in the field. First, there are serious practical difficulties in measuring dose and response in the field, which will be addressed in the next section. Second, the field situation is likely to be complicated by other factors that are not under the control of the experimenter. The presence of other chemicals and other organisms and changes in temperature, pH, wind speed, and rate of flow of water may all have effects on a dose-response relationship. Thus, knowledge and/or control of environmental factors are important when making comparisons between laboratory and field. The use of mesocosms (e.g., artificial ponds, simulated streams) may provide a halfway point between the two extremes where

dose-response relationships may be tested (12). Here, to a considerable extent, environmental factors are under the control of the experimenter. Also, knowledge of potentially complicating environmental factors may lead to the design of more sophisticated laboratory studies. Laboratory studies may be designed to model real environmental conditions more closely. For example, other environmental chemicals may be introduced into the test system to discover whether potentiation occurs (as discussed in "Biomarkers and Interactive Effects"); temperatures and pH values may be altered to see whether they affect the dose-response relationship.

A major difficulty with laboratory and field comparisons is that it is only possible to study a tiny proportion of the species of interest in the laboratory. Thus, the selection of sensitive or key species that can be studied in the laboratory is a critical consideration. Species that are widely distributed in polluted environments and are representative of major groups of animals are logical choices. Examples of such species include starling (*Sturnus vulgaris*), red-legged partridge (*Alectoris rufa* cross), and pigeon (*Columba livia*) as representatives of birds; edible mussel (*Mytilus edulis*) as a representative of mollusks; and crabs (*Carcinus* spp.) as representatives of crustaceans (13-15). Sometimes dose-response relationships for biomarker assays that measure toxicity do not vary greatly between species [e.g., brain acetylcholinesterase inhibition in birds (6)]. A dose-response curve for one species may be applicable to many other related species in the field. Unfortunately this is often not the case [e.g., relationship between dichlorophenyl dichloromethylene (DDE) and eggshell thinning in birds (1)].

A recent novel approach to the problem under discussion is the use of *in vitro* biomarker responses to aid the process of environmental risk assessment. A pertinent example is the use of a transfected cell line that has a reporter gene e.g., the chemically activated luciferase gene expression (CALUX) system, which will be discussed in "Use of Cell Cultures" (16). This kind of system can be useful for providing an integrated response of a biochemical site of action after exposure to a mixture of related chemicals.

Biomarker Strategies in the Field

In environmental risk assessment, comparisons are made between measured or estimated environmental concentrations and estimated environmental toxicity (17).

Both of these elements are extremely hard to determine with any degree of accuracy. The objective of the biomarker approach, on the other hand, is to give evidence of environmental exposure and consequent toxic effects. The extent to which an organism is actually exposed to a known environmental concentration comes into question. This is relatively easy to address for aquatic organisms when a pollutant is dissolved in water. It is harder to determine for residues in sediments because their availability is often uncertain. In the terrestrial environment, a residue of a pesticide on a seed may or may not be taken up by a bird. Much depends on the feeding behavior of the bird.

When investigating dose-response relationships in the living environment, there are, broadly speaking, two approaches: the study of dose-response relationships that already exist or causing the exposure of organisms to pollutants by direct intervention. In the first case, biomarker responses can be estimated along pollution gradients by making comparisons between polluted organisms and clean organisms from similar

sites (controls). A major difficulty of such an approach is identifying sites that are truly clean and representative of polluted areas. The other approach can be more rigorously controlled and involves manipulation of either the organism or the chemical. One strategy is to deploy clean organisms into both polluted and control sites. Biomarker assays may then be performed both before and at different times after deployment (18). Biomarker responses are then calculated by comparing results from assays for polluted sites with those for the controlled site and plotting a dose-response curve. Another strategy is to deliberately release the pollutant into the environment while keeping a control area free of chemical. Biomarker assays can then be performed before and at different times after the release of the chemical, and comparisons made between samples from control and treated areas to establish biomarker response. The latter approach has been used in field trials with pesticides e.g., the Boxworth experiment (19,20). With these controlled studies it is possible, in theory, to have properly

replicated experiments. However, this depends on circumstances, and with relatively extensive studies involving vertebrates may not be practically possible (20).

Biomarker Assays

In the following sections, some biomarker assays that have already enjoyed application or are at an advanced stage of development will be considered. Their attributes will be reviewed in the light of concepts discussed in the previous sections. Separate sections will be devoted to molecular, cellular, and whole-organism biomarkers.

Molecular Biomarker Assays

Vertebrates. ASSAYS USING BLOOD. It is relatively easy to obtain blood samples from most vertebrate animals, and there are already an impressive number of biomarker assays that can be performed on blood (Table 1).

Both cholinesterases and carboxylesterases are examples of 'B' esterases that occur in blood. OPs act as suicide substrates, phosphorylating serine residues that

Table 1. Biomarker assays for blood.

Biomarker	Type of environmental chemical	Biomarker of toxic effect	Characteristics	Reference
Cholinesterase inhibition	OPs, carbamate	No	Have been used as biomarkers of exposure. Variability of normal levels a problem. Recent immunochemical assays overcome this problem but are not available commercially.	(21-24)
Carboxylesterase inhibition	OPs, carbamate	No	Same comments as above except that they have not been widely used.	(21,22,25)
Increase in precursors of blood clotting protein (descarboxy coagulation protein)	Vitamin K antagonists such as warfarin and related rodenticides	Yes	ELISA assays have been used in humans exposed to warfarin. Not known to what extent assay works in other species.	(26)
Fall in retinol (vitamin A) and thyroxine (T ₄) levels, and reduction of available T ₄ binding sites of transthyretin	Hydroxymetabolites of certain PCBs, especially 4-OH-3,3',4,5'-TCB.	Yes	Have been used successfully on mammalian species including seals. Results with birds variable. Assay of T ₄ binding sites requires specialized laboratory.	(27,28)
Inhibition of ALAD	Lead	No	Good specific test for exposure to lead. Has been widely used and is readily available.	(1)
Changes in porphyrin levels	A number of compounds, including halogenated compounds such as HCB, 3,3',4,4'-TCB and TCDD	In some cases	Not very specific. Some potential for use in the field.	(30)
Formation of DNA adducts (white blood vessels)	PAHs and other environmental mutagens, including oxyradicals	Not at present state of knowledge	Several techniques including 32P post-labelling. HPLC/fluorescence and ELISA have been used in environmental studies. Possibility of detection by DNA fingerprinting.	(29,31)
DNA strand breaks	PAHs and other environmental mutagens, including oxyradicals	Not at present state of knowledge	Alkaline unwinding assay and Comet assay have been used in environmental studies.	(29)
Formation of hemoglobin adducts	PAHs and other environmental mutagens, including oxyradicals	No	HPLC/fluorescence, GC/MS, and other complex analytical techniques.	(29)
Vitellogenin production in male fish	Estrogens	Yes	Determined by radioimmunoassay. Not specific for particular contaminants.	(32)

Abbreviations: GC/MS, gas chromatography/mass spectroscopy; HCB, hexachlorobenzene; HPLC, high performance liquid chromatography; TCB, tetrachlorobiphenyl; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

exist at their active centers and so cause virtually irreversible inhibition (21,22). Depression of blood 'B' esterase activities can last for several days after exposure in mammals and birds and considerably longer in one species of lizard (23). Inhibition provides a valuable biomarker of exposure to OPs and carbamates in field studies (22), but does not give a reliable indication of toxic effect (compare brain acetylcholinesterase, which represents the site of action of these compounds). A limitation of these assays is the variability of esterase levels in blood, making control values hard to establish. Recent work concerns the development of ELISA assays, which allow the estimation of specific activity, thus avoiding this problem and facilitating more sensitive detection of esterase inhibition (24,25).

Anticoagulant rodenticides such as warfarin act as vitamin K antagonists, thereby inhibiting the completion of synthesis of clotting proteins in the liver. The precursors of clotting proteins are released into the blood instead of the fully synthesized proteins. Over a period of days, the levels of clotting proteins in blood fall until the blood will no longer clot and hemorrhaging occurs. ELISA assays exist for the determination of a precursor of a clotting protein in human blood (26). It is not yet known whether the same assay is effective in wild vertebrates of interest in ecotoxicology. In principle, however, this could provide an excellent biomarker of toxic effect for use in field studies. Currently, there is concern about the possible effects of new rodenticides related to warfarin (e.g., brodifacoum and flocoumafen) on predators and scavengers that feed on rodents (e.g., owls and corvids).

Another example of blood biomarker assays that measure toxic effects relates to thyroxine (T_4) antagonism of the PCB metabolite 4'-hydroxy-3,3',4,5'-tetrachlorobiphenyl. This, and to a lesser extent other PCB metabolites, can compete with T_4 for binding sites on the blood protein transthyretin. When T_4 is displaced, transthyretin dissociates from an associated retinol-binding protein. Consequently both T_4 and retinol (vitamin A) are lost from blood (27,28). A number of toxic effects may result, including certain symptoms of vitamin A deficiency. Three biomarker assays have been used to monitor this toxic interaction: reduction of blood T_4 ; reduction of vitamin A; and reduction of the number of free T_4 binding sites on transthyretin. These assays have been used successfully in a number of mammalian

species, including seals. However, results with birds have been variable; some species apparently do not show the same mode of action as mammals (28).

There has been considerable success in developing assays for genotoxicity in medical toxicology. PAHs, for example, form adducts with the DNA of white blood cells (using techniques such as ^{32}P postlabeling) and also with hemoglobin of red blood cells (29). Assays have also been developed for measuring DNA strand breaks (alkaline unwinding assay and Comet assay). These assays are being used in ecotoxicologic studies, albeit usually on tissues such as liver after destructive sampling. Although some adducts can lead to mutation and consequent toxic damage e.g., carcinogenesis, there is not yet a clear relationship between this biomarker assay and toxic effect.

Briefly, exposure to lead can be measured by aminolevulinic acid dehydratase inhibition and changes in blood porphyrins (30) can be caused by a number of chemical agents (Table 1).

SKIN AND OTHER TISSUES TAKEN BY BIOPSY. Both residues of persistent chemicals and cytochrome P450 have been measured in samples of skin taken from animals. Currently this approach is being followed in a study of dolphins from which skin samples are taken by a dart (33). In principle it is also possible to sample other tissues by biopsy e.g., liver samples, to perform biomarker assays. The assays for genotoxicity mentioned above may be performed on such samples. Levels of stress proteins can be determined in various tissues.

EGGS. Eggs of birds, amphibians, and reptiles can be sampled to perform biomarker assays, a practice that is usually regarded as nondestructive. Retinol (vitamin A) levels in eggs can be affected by chlorinated organic compounds and PAHs (1).

ANALYSIS OF EXCRETA. Changes in profiles of excretory products can be determined by various analytical procedures in samples of feces and urine. Such changes are relevant to the present discussion when they are caused by the action of environmental chemicals. A pertinent example is the determination of changes in porphyrin patterns in urine or feces by high performance liquid chromatography following exposure to chemicals (32). Nuclear magnetic resonance spectroscopy has also been used to detect changes in urinary components (34,35).

Invertebrates. Molecular biomarker assays in invertebrates have been implemented to a limited extent. Much of the

early work with them has involved the adaptation of biomarker assays (e.g., induction of cytochrome P450), which are well established in vertebrates. However, it has become rapidly apparent how invertebrates can differ and vertebrates (35), and there is clearly a need for more fundamental work on the biochemical toxicology of invertebrates (aquatic and terrestrial) if this field is to prosper. Work of this kind has been conducted with the edible mussel (*M. edulis*). Studies with digestive gland microsomes have produced evidence of induction of a form of cytochrome P450 by PAH (36).

Studies have also been done with *Carcinus* spp. (15). Exposure to benzo[a]pyrene (B[a]P) caused an increase in the B[a]P monooxygenase activity in microsomes from the hepatopancreas; this was associated with an increase of a protein band (48 kDa) resolved by sodium dodecyl sulfate-polyacrylamide gel. Purification of this protein has been carried out to determine if it is a form of cytochrome P450. A major objective of this work is to obtain an antibody to an individual form of P450 that can then be used in an immunochemical biomarker assay for *Carcinus* spp. and other aquatic invertebrates.

There have also been reports of DNA adduct formation by *Mytilus galloprovincialis* when exposed to PAH (37).

Use of Cell Cultures. This is a relatively new approach to the use of biomarker strategies. An example of particular interest and importance addresses the problem of the combined effects of mixtures of organohalogen compounds that cause aryl hydrocarbon (Ah) receptor-mediated toxicity (16,38-40). One of the most widely used destructive biomarker assays is the induction of hepatic microsomal cytochrome P450 1A1 and associated activity (ethoxyresorufin deethylase) caused by coplanar PCBs, polychlorinated dibenzodioxins (dioxins), polychlorinated dibenzofurans, and certain PAHs. This induction follows the interaction of the pollutants with the so-called Ah receptor, and is one of a number of responses associated with the operation of the Ah receptor signaling pathway. A number of toxic responses (e.g., disturbances of thyroid and sex hormones, changes in levels of vitamins A and C) are also associated with the stimulation of this pathway. Certain hepatoma cell lines (e.g., mouse Hepa-1c 1c 7' and rat H 4.IIe) contain the Ah receptor. Recently such cells have been transfected with reporter genes (16,40). One example is the

CALUX system (16). Very small quantities of organohalogen compounds added to this system can cause the synthesis of luciferase via an Ah receptor-dependent pathway, and this leads to the emission of photons. This system can give a measure of the integrated effect of mixtures of organohalogen compounds that interact with the Ah receptor.

A number of studies have demonstrated induction of P450s in fish hepatocytes, including those of rainbow trout (*Salmo Gairdneri*) and flounder (*Platychthys flesus*) (41–43). A recent example of this approach involves the use of primary culture of salmon (*Salmo salar*) hepatocytes exposed to pollutants (44). Responses were measured by ³⁵S-methionine/cysteine incorporation and Western blotting. There was evidence for induction of a stress protein by four different pollutants: B[a]P, 2,3,3',4,4'-pentachlorobiphenyl, AsO₂⁻, and Cd. The two organic pollutants induced cytochrome P450 1A1 in a dose-dependent manner. Also, primary cultures of trout hepatocytes have been used to measure the activity of environmental estrogens (32). The measured response is an increase in vitellogenin.

Certainly cell systems hold considerable promise for the future and can provide biomarker assays based on the mode of toxic action. Clearly, however, there are problems in extrapolating from responses such as these to toxic effects on living organisms exposed to the same levels of chemicals.

Microorganisms. The Microtox test system (Microbics Corp., Carlsbad, CA), which employs the bioluminescent marine bacterium *Vibrio fischeri*, is commercially available and widely used (45). Emission of light by the bacterium is regulated by a form of luciferase and toxicity is measured by the degree of inhibition of light emission.

It should also be mentioned that bacterial mutagenicity assays such as the Ames test can be used to detect the presence of mutagens in environmental samples, including extracts of tissues and eggs.

Structural Changes. Where samples of tissues can be obtained, structural changes caused by chemicals can be observed by microscopy and electron microscopy, including proliferation of endoplasmic reticulum, lysosomal damage, mitochondrial damage, hypertrophy, and hyperplasia.

Two of the most valuable biomarkers yet discovered in the context of ecotoxicology are based on structural change. When organochlorine insecticides were widely used, it was discovered that *p,p'*-DDE, a persistent metabolite of the insecticide

p,p'-dichlorophenyltrichloroethane, could cause eggshell thinning in certain species of birds. This effect is caused by a reduction in the transfer of Ca²⁺ in the shell gland, probably because of inhibition of Ca²⁺ adenosine triphosphatase. Species such as the peregrine falcon (*Falco peregrinus*), the sparrowhawk (*Accipiter nisus*), and the Gannet (*Sula bassana*) all experienced eggshell thinning due to environmental levels of *p,p'*-DDE. In some cases this caused a decline in reproductive success, leading to population decline (1). In another example, very low levels of TBT in coastal waters caused imposex in female dogwhelks. The development of a penis caused blockage of oviducts and reproductive failure. Population decline of the dogwhelk was widely reported in Britain and other Western European maritime countries as a result of this effect (7). As in the earlier example, a physiologic biomarker response gave critical evidence, causally linking the presence of an environmental chemical to population decline in a dose-dependent fashion.

Tests of Cellular Function. In *M. edulis*, lysosomal damage caused by pollutants has been studied using a physiologic test (46). The action of mitochondrial poisons (e.g., uncouplers such as chlorinated phenols, rotenone) can be established by performing controlled studies on respiration by isolated mitochondria.

Studies on the Whole Organism

With vertebrates, harmful effects of chemicals on the function of the whole organism can be measured using physiologic and behavioral assays. Respiration, cardiac function, blood flow, and neuroactivity can be monitored using techniques established by medical scientists.

In the case of neurotoxic pollutants, there is particular interest in behavioral changes caused by sublethal effects on the nervous system. It is worth emphasizing that many poisons—including all four major groups of insecticides—act as neurotoxins. OPs, for example, can cause changes in the behavior of vertebrates at levels of exposure well below those that cause death (6). In birds, movement, feeding behavior, and singing have been affected. In the context of ecology, disturbances of feeding or reproduction can be more harmful to the species than the straightforward lethal toxicity of chemicals.

In invertebrates, the effects of chemicals on the whole organism have sometimes been measured as changes in scope for growth. This concept is based on an assessment of

the surplus energy that an organism has for growth and reproduction after accounting for the basic requirements for respiration, tissue repair, and other functions (47). Chemical stress will bring an energy cost to an organism e.g., for mounting defense systems or repairing tissue damage caused by the chemical. The larger this cost is, the less that can be invested in growth or reproduction.

Recently other assays have been developed for invertebrates. In *Carcinus* spp., for instance, a cardiac monitor has been developed (15,48). Detailed analysis of this has produced evidence of characteristic responses to certain types of metal pollution.

Conclusions

In the foregoing account, biomarker strategies were reviewed with the ultimate objective of using them to obtain clear evidence of toxic effects of environmental chemicals on animals (especially at the level of population and above) while minimizing harm to vertebrates during the course of the testing procedures. There is the immediate problem that some of the most valuable biomarker assays currently used depend on destructive sampling. Examples include inhibition of brain acetylcholinesterase and (unless available by biopsy) the induction of liver enzymes such as P450 1A1. Because of the large differences that exist among species in their response to pollutants, extrapolation of data from one species to another is no easy matter. This problem is recognized in environmental risk assessment where a large safety factor is used in the estimation of environmental toxicity from laboratory data (17). Thus the use of nondestructive biomarker assays in other species actually exposed to pollutants in the field provides the most clear cut and objective approach to the problem. Using strategies described earlier, harmful effects of pollutants on individuals can be measured in the field and attributed to a particular chemical, or combination of chemicals, in a dose-dependent manner. Data so obtained can then be used to establish causal relationships between levels of pollutants and effects at the population level. The effectiveness of this approach has already been demonstrated in the case of eggshell thinning caused by *p,p'*-DDE in birds of prey and in imposex caused by TBT in the dogwhelk (1,7).

Although attractive in theory, such an approach is limited by the relatively small number of available nondestructive assays. Notwithstanding this general observation, there is already a promising collection of

assays that can be used in blood, which is relatively easy to obtain from certain species in the field. Although little used as yet, there is considerable potential for the development of assays using excreta and tissues taken by biopsy. The eggs of birds, amphibians, and reptiles are readily obtained and could be much more widely used for biomarker studies.

A very interesting, although largely undeveloped, area is the use of *in vitro* methods. The CALUX system, which can measure the interaction of combinations of chemicals with the Ah receptor, is a pertinent example (38,40). Cell cultures derived from species being studied in the field can be used. These may carry the sites for action and certain of the detoxifying/activity enzymes relevant to particular types of pollutant. However, the extrapolation of *in vitro* data to *in vivo* is not a simple matter even for the same species (9). *In vitro* methods may give valuable information on hazards of environmental chemicals to wild vertebrates, but they cannot be expected to give reliable predictions of effects on individuals, let alone populations, with our present state of knowledge.

Biomarker assays for invertebrates are still in an undeveloped state. Whereas rapid

progress in medical toxicology has paved the way for biomarker assays in other vertebrates, invertebrates have proved very different in critical aspects of their biochemistry and physiology, making progress difficult and slow. For example, antibodies raised to mammalian forms of cytochrome P450 did not recognize P450s of certain invertebrates (15). Also, the funding for work on invertebrates has been very small in comparison to the support given to biomarker studies in medical toxicology. Biomarker studies on invertebrates are of particular importance when considering effects of chemicals on communities and ecosystems because of their strong representation in lower trophic levels. Their value in measuring or predicting effects of chemicals on invertebrates is, however, limited. Because they are so different biochemically, they cannot be regarded as simple surrogates for vertebrates when considering biomarker responses related to toxic effect. They can, however, provide useful indications of exposure of vertebrates to pollutants in the natural environment. In aquatic ecosystems they can give indications of available pollutants in the water column. They also give some indication of the residue burden that will be passed on to vertebrates that prey on them.

In conclusion, nondestructive biomarker assays on vertebrates that make appropriate comparisons between laboratory and field provide a logical approach to the assessment of the environmental effects of pollutants, which has already proved its worth. Such assays are of most value when they can be used across a range of species. Specificity for a particular type of chemical is desirable; for a particular species it is too limiting. *In vitro* assays with vertebrate material hold great promise, but are at an early stage of development and will always raise the problem of extrapolation to *in vivo*. Finally, invertebrate biomarker assays are of fundamental interest in regard to assessment of pollutant effects at the levels of community and ecosystem. They are potentially valuable for giving measures of exposure of vertebrates to pollutants in the natural environment. The development of biomarkers for invertebrates and *in vitro* biomarkers for vertebrates is at an early stage. Biomarkers have the potential to greatly advance our knowledge of the environmental effects of chemicals on animals. However, this would require considerable investment as part of a long-term strategy.

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